IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **Jon A. Wolff,**)
Vladimir S. Trubetskoy,)
Sean D. Monahan, James E. Hagstrom,)
Paul M. Slattum, Vladimir G. Budker,)
Aaron G. Loomis)
)
Serial No.: **09/328,975**)
) Examiner: **Richard Schnizer**
Filed: **6/9/99**)
)
Group Art Unit: **1632**)

For: **Charge Reversal of Polyion Complexes**

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, DC 20231

Dear Sir:

I, Vladimir Trubetskoy, hereby declare as follows:

1. I am an inventor of the captioned application.

2. The inventors submit with this Declaration and Response further experimental material illustrating *in vivo* delivery and expression of recharged nucleic acids.

3. The material is consistent with the specification as filed and the methods described have been used as shown.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____

Vladimir Trubetskoy Date

Additional example on in vivo application of recharged complexes.

The recharged triple complex DNA/linear PEI(lPEI)/polyacrylic acid (pAA) containing β-galactosidase-expressing pCILacZ plasmid was prepared in 10 mM HEPES, 0.29 M glucose, pH 7.5 at 1:4:1 weight ratio at the DNA concentration of 0.2 mg/ml.

A DNA expression vector containing the gene for β-galactosidase was delivered to biliary epithelial cells via the sealed end of the tubing, which was opened and the solutions were injected under low pressure. 24 hours after total bile duct obstruction with sealed polyethylene tubing, the tubing was opened, letting the bile leak out, connected to 31 G needle and 0.12-0.14 ml of DNA/PEI/pAA recharged complexes were infused over 5 seconds. The syringe remained connected to the tubing for 10 minutes and the infusion was repeated at 10 and 20 minutes after the first infusion, reaching a total amount of injected solution of 0.4 ml. Then tubing was sealed with hot forceps prior to syringe disconnecting.

Staining for β-galactosidase activity was employed to determine the cells transfected with this complex. In brief, 24 hours' posttransfection with pCILacZ gene, the animals were sacrificed. Immediately after the animals were euthanized, the livers were harvested and embedded in O.C.T. compound and snap-frozen. 5-7 micrometers frozen sections were prepared using the cryostat Microm HM 505 N from (Carl Zeiss, Goettingen, Germany), mounted on the charged precleaned slides (Fisher Scientific) and air dried overnight at room temperature. Before β-galctosidase staining the slides were fixed in 2% formaldehyde for 10 minutes. Then sections were washed three times with PBS, and incubated for 4 hours in a solution containing 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl--D-galactopyranoside), 25 mmol/L $K_3Fe(CN)_6$, 25 mmol/L K4Fe(CN)6 (3H2O), and 1.5 mmol/L MgCl2 in PBS at 37°C. Blue-stained cells expressing the β-galactosidase gene were examined under Axioplan-2 microscope and pictures were taken with the aid of AxioCam digital camera (both from Carl Zeiss, Goettingen, Germany). Portal areas in non-counterstained sections were determined by finding two and more adjoining vessel-like structures.

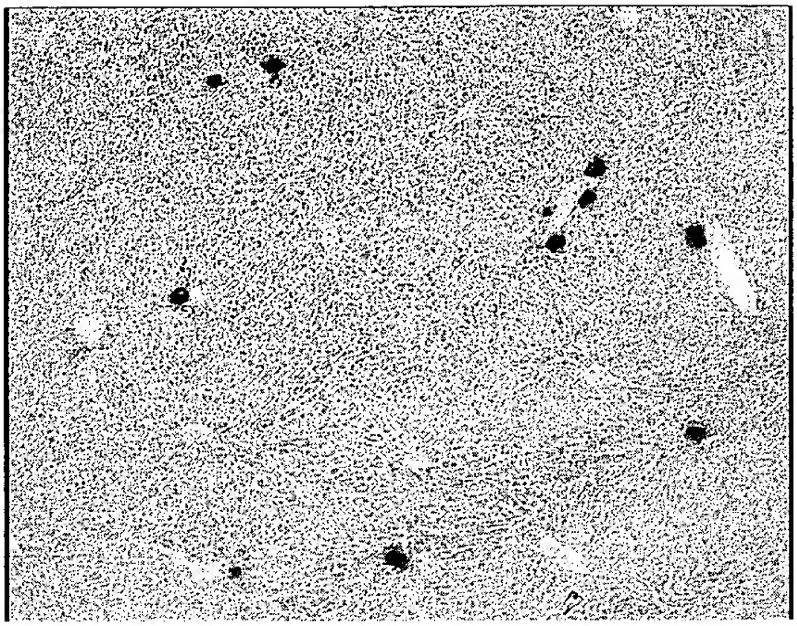

Fig. 1. Frozen section of the liver one day after total bile duct ligation, DNA/lPEI/pAA tranfections were carried out 24 hours' post-bile duct ligation using the pCILacZ reporter plasmid. Only biliary epithelial cells adjoining to portal areas structures are stained with X-gal. Magnification x100.

Examination of X-gal- stained section revealed that 10-50% of portal triads (depending on area of section) included blue-stained cell groups expressing β-galactosidase gene. The gene delivery efficiency was such that in some areas it was extremely rare to find two and more adjoining vessel-like structures without staining. In some stained structures a low-dense staining appeared in the center, suggesting a leaking of stain into the bile duct lumen, indirectly confirming a specific targeting of bile ducts.